



## Induction of G<sub>2</sub>/M arrest, caspase activation and apoptosis by $\alpha$ -santonin derivatives in HL-60 cells

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### ABSTRACT

Sesquiterpene lactones (SLs) are natural products with a variety of biological activities. Previously, we demonstrated the cytotoxic effects of three new  $\alpha$ -santonin derivatives on different tumor cell lines with low toxic effects upon peripheral human leukocytes. Here, we evaluated the mechanism of action triggered by these derivatives. HL-60 cell cycle determined after 24 h treatment revealed a significant inhibition on cell-cycle progression and leading to an increasing of cells in G<sub>2</sub>/M [7.6% and 9.0% for compound 3% and 9.0% and 8.6% for compound 4 (1 and 2  $\mu$ M, respectively)]. However, after 48 h exposure, all compounds caused G<sub>2</sub>/M reduction and a significant DNA fragmentation. Compounds 2, 3 and 4 were able to induce depolarization of the mitochondrial membrane potential, which was associated with externalization and apoptosis. These results suggest that  $\alpha$ -santonin derivatives induce apoptosis through the activation of the intrinsic apoptotic pathway in the death process. The antiproliferative action of these compounds is related to the DNA synthesis inhibition and cell cycle arrest, which probably lead to apoptosis activation. Therefore, these santonin derivatives are promising lead candidates for development of new cytotoxic agents.

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### 1. Introduction

Cancer is a disease caused by disorderly growth of cells that often invade tissues and organs. Considerable insight has been gained into the mechanisms by which some chemicals affect cellular growth and this knowledge has been used to design new more selective chemotherapeutic drugs towards cancer cells than to normal cells and reduce side effects (Benz and Yau, 2008).

The development of antineoplastic agents is important to diminish the mortality caused by cancer. Since cell homeostasis depends on the balance between proliferation and cell death, effective compounds that increase apoptosis without concomitant increases cellular proliferation appear to be a relevant strategy to suppress

tumor growth (Kaufmann and Earnshaw, 2000; Ferreira et al., 2010).

Despite the interest in molecular modeling and combinatorial chemistry, the search for novel anticancer drugs from natural and non-natural sources has continued through the collaboration of scientists worldwide in looking for new bioactive compounds (Kiran et al., 2008; Cragg et al., 2009; Ferreira et al., 2011). Among the large sources of potential compounds natural products offer opportunities to evaluate not only totally new chemical classes of anticancer agents, but also novel and potentially relevant mechanisms of action. The majority of anticancer drugs are natural products or their derivatives and more than 200 drugs derived from natural products are in preclinical or clinical development and evaluation (Ghantous et al., 2010; Newman and Cragg, 2012).

Sesquiterpene lactones (SLs) are a class of naturally occurring plant terpenoids of the Asteraceae family, known for their various biological activities such as anti-inflammatory, phytotoxic, antimicrobial, antiprotozoal, and cytotoxic against different tumor cell lines (Hehner et al., 1998; Mazon et al., 2000; Schmidt et al.,

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2002; Zhang et al., 2005).  $\alpha$ -Santonin, a sesquiterpene lactone isolated from *Artemisia santonica* presents antipyretic, anti-parasitic and anti-inflammatory properties (Ivasenko et al., 2006). Some  $\alpha$ -santonin derivatives also act as inhibitors of phospholipase A<sub>2</sub> enzymes from *Bothrops jararacussu* (De Alvarenga et al., 2011). Additionally, we have reported the activity of synthetic  $\alpha$ -santonin derivatives against several human cancer cell lines (HL-60, leukemia; SF-295; glioblastoma; HCT-8, colon; MDA/MB-435, melanoma) with low antiproliferative effects upon normal human leukocytes (Arantes et al., 2009, Arantes et al., 2010). Therefore, these results indicate that SLs and related compounds may represent a promising class of biological agents. In this work, we described, for the first time, the mechanism of induction of cell death on human promyelocytic leukemia HL-60 cell line triggered by three  $\alpha$ -santonin derivatives.

## 2. Methods

### 2.1. Chemicals

Fetal calf serum was purchased from Cultilab (Campinas, SP), RPMI 1640 medium, trypsin–EDTA, penicillin and streptomycin were purchased from GIBCO® (Invitrogen, Carlsbad, CA, USA). Propidium iodide (PI), acridine orange (AO), ethidium bromide (EB) and Rhodamine 123 (Rho-123) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Doxorubicin (Doxolem®) was purchased from Zodiac Produtos Farmacêuticos S/A, Brazil. All other chemicals and reagents used were of analytical grade.  $\alpha$ -Santonin (compound 1) (97%) was procured from Sigma–Aldrich Co. (Milwaukee, WI, USA) and was utilized without further purification. The transformation of  $\alpha$ -santonin (compound 1) into lactone (compound 2), and its further transformation into (compound 3) and (compound 4) were carried out as previously described (Arantes et al., 2010) (Fig. 1).

### 2.2. Study of the mechanisms involved in the cytotoxic activity on HL-60 cells

Since HL-60 cell line was the most sensitive cell line to  $\alpha$ -santonin derivatives (Arantes et al., 2010), it was selected to evaluate the mechanism underlying to their cytotoxic effects after 24 h exposure. The compounds dissolved in DMSO (0.1%) were added to cell cultures of HL-60 cells ( $3 \times 10^5$  cells/mL) to obtain final concentrations of 1 and 2  $\mu$ M. Doxorubicin (0.6  $\mu$ M) was used as a positive control (Dox). All flow cytometry analyses were performed in a Guava® EasyCyte Mine using Guava Express Plus CytoSoft 4.1 software (Guava Technologies Inc., Industrial Blvd., Hayward, CA, USA). Five thousand events were evaluated per experiment and cell debris was omitted from the analysis.

#### 2.2.1. Trypan blue exclusion test

Cell viability was determined by the trypan blue dye exclusion test (Kepp et al., 2011). The cell samples were diluted in trypan blue dye of an acid azo exclusion medium by preparing a 1:1

dilution of the cell suspension using a 0.4% trypan blue solution. Non-viable cells were labeled in blue and are visible with bright-field optics and viable cells were unstained, since viable cells maintain the capacity to extrude this vital dye. The count was performed under the microscope in four  $1 \times 1$  mm squares of a Neubauer chamber. Number of cells ( $\times 10^4$  cells/mL) was stated and viable and non-viable cells were expressed as a percentage of total cells.

#### 2.2.2. Inhibition of DNA synthesis detected by immunocytochemistry

Cells were plated in 24-well tissue culture plates (2 mL/well) and treated with the compounds. After 21 h exposure, 20  $\mu$ L of 5-bromo-2'-deoxyuridine (BrdU, 10 mM) was added and incubated for 3 h at 37 °C. To determine the amount of BrdU incorporated into DNA (Pera et al., 1977), cells were harvested, transferred to cytospin slides, and allowed to dry for 2 h at room temperature (25 °C). Cells were labeled using direct peroxidase immunocytochemistry by the chromogen diaminobenzidine (DAB) staining those cells that incorporated Brd. Slides were counterstained with hematoxylin and coverslipped. The determination of BrdU positivity was performed by light microscopy (Olympus, Tokyo, Japan). Two hundred cells were counted per sample to determine the percentage of BrdU-positive cells (Costa et al., 2008).

#### 2.2.3. Morphological analysis with fluorescence microscopy

To determine whether the growth inhibition activity of compounds 2–4 was related to the induction of apoptosis or necrosis, morphological analysis of treated cells was investigated by fluorescent microscopy using acridine orange/ethidium bromide (AO/EB) staining. After 24 h incubation, cells were pelleted and each sample was mixed with 1 L of aqueous AO/EB solution (100 g/mL of AO in PBS; 100 g/mL EB in PBS) just prior to fluorescence microscopy and quantification (Olympus, Tokyo, Japan). Three hundred cells were counted per sample and scored as follows: viable cells, apoptotic cells and necrotic cells (Cury-Boaventura et al., 2004; Tamatani et al., 2012). The percentage of apoptotic and necrotic cells was then calculated.

#### 2.2.4. Cell cycle distribution and internucleosomal DNA fragmentation analysis

Cell cycle distribution and DNA fragmentation analysis were evaluated by the incorporation of propidium iodide (50  $\mu$ g/mL). Briefly, 24 h-treated and untreated cells ( $3 \times 10^5$  cells/mL) were incubated at 37 °C for 30 min in the dark, in a lysis solution containing 0.1% citrate, 0.1% Triton X-100 and 50  $\mu$ g/mL propidium iodide and fluorescence was measured afterwards.

#### 2.2.5. Cell membrane integrity

Cell membrane integrity was evaluated by the exclusion of propidium iodide. Briefly, 100  $\mu$ L of treated and untreated cells were incubated with propidium iodide (50  $\mu$ g/mL). The cells were then incubated for 5 min at 37 °C. Fluorescence was measured and cell morphology, granularity and membrane integrity were determined (Darzynkiewicz et al., 1992).

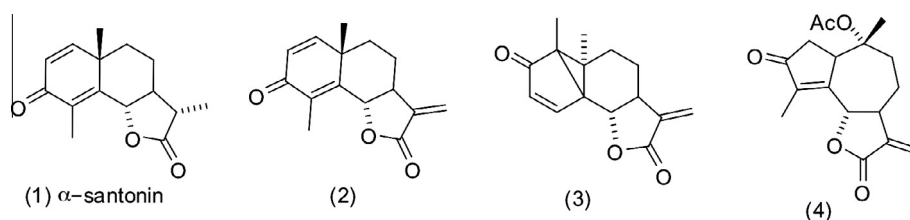


Fig. 1. Structures of  $\alpha$ -santonin (1) and its synthetic derivatives (2–4).

### 2.2.6. Phosphatidylserine (PS) externalization

PS externalization was analyzed by flow cytometry (Annexin V) according to [Vermes and co-workers \(1995\)](#) using Guava Nexin Assay Kit. Briefly, cells ( $3 \times 10^5$  cells/mL) were washed twice with cold PBS and then resuspended in 135  $\mu$ L of PBS with 5  $\mu$ L of 7-aminocincomycin D (7-AAD) and 10  $\mu$ L of Annexin V-PE. Cells were gently vortexed and incubated for 20 min at room temperature ( $22 \pm 2$  °C) in the dark. Afterwards, cells were analyzed by flow cytometry (EasyCyte from Guava® Technologies). Annexin V is a phospholipid-binding protein that has a high affinity for PS. 7-AAD, a cell impermeant dye, is used as an indicator of membrane structural integrity. Fluorescence of Annexin V-PE was measured in yellow fluorescence-583 nm and 7-AAD in red fluorescence-680 nm. The percentage of early and late apoptotic cells and necrotic cells was then calculated.

### 2.2.7. Detection of caspase 3 and 7

Active catalytically caspases-3/7 were analyzed by flow cytometry using Guava® EasyCyte Caspase Kit after 24 h of incubation. HL-60 cells ( $3 \times 10^5$  cells/mL) were incubated with Fluorescent Labeled Inhibitor of Caspases (FLICAs) and maintained for 1 h at 37 °C and 5% CO<sub>2</sub>. After incubation, 80  $\mu$ L of washing buffer were added and cells were centrifuged at 2000 rpm for 5 min. The resulting pellet was resuspended in 200  $\mu$ L of washing buffer and centrifuged again. Then, cells were resuspended in the working solution (propidium iodide 1:200 in 1 $\times$  washing buffer) and analyzed immediately by flow cytometry.

### 2.2.8. Measurement of mitochondrial transmembrane potential ( $\Delta\psi_m$ )

Mitochondrial transmembrane potential was determined by rhodamine 123 dye retention using flow cytometry. Rhodamine 123 is a cell-permeable, cationic, fluorescent dye that is readily sequestered by lively mitochondria without inducing cytotoxic effects. Cells ( $3 \times 10^5$  cells/mL) were washed with PBS, incubated with rhodamine 123 at 37 °C for 15 min in the dark. Cells were incubated again in PBS at 37 °C for 30 min in the dark, and fluorescence was measured ([Militão et al., 2006](#)).

### 2.3. In vitro alkaline comet assay

Heparinized blood was collected from healthy, non-smoker donors who had not taken any medication for at least 15 days prior to sampling and with no history of recent exposure to potentially genotoxic substances (i.e., pesticides, drugs, alcohol, tobacco or ionizing radiation, such as X-rays). All studies were performed in accordance with Brazilian (Law 196/96, National Council of Health) and international (Declaration of Helsinki) guidelines.

Human peripheral blood mononuclear cells (PBMCs) were isolated by the standard method of density-gradient centrifugation over Histopaque-1077. Cells were washed and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin, at 37 °C under 5% CO<sub>2</sub>. Phytohemagglutinin (4%) was added at the beginning of culture. After 24 h of culture, PBMC were treated with the test substances.

The alkaline comet assay was performed as described by [Singh et al. \(1988\)](#) with minor modifications ([Hartmann and Speit, 1997](#)), and following the recommendations of the International Workshop on Genotoxicity Test Procedures ([Tice et al., 2000](#)). At the end of the treatment, cells were washed with ice-cold PBS, detached with 100  $\mu$ L trypsin (0.15%) and resuspended in complete RPMI medium. Next, 20  $\mu$ L of cell suspension ( $\sim 10^6$  cells/mL) were mixed with 100  $\mu$ L of 0.75% low melting point agarose and immediately spread onto a glass microscope slide precoated with a layer of 1% normal melting point agarose. Agarose was allowed to set at 4 °C

for 5 min. The slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4 °C for a minimum of 1 h to remove cellular proteins, leaving the DNA as “nucleoids”. After the lysis procedure, the slides were placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13.0) to cover the slides for 20 min at 4 °C to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was carried out for 20 min at 25 V and 300 mA (0.86 V/cm). After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with ethidium bromide (20  $\mu$ g/mL) and analyzed using a fluorescence microscope. All the above steps were conducted under yellow light or in the dark to prevent additional DNA damage. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each concentration of test substance. Cells were scored visually and classified in 5 grades according to the tail size (from undamaged-0 to maximally damaged-4), and a damage index value was calculated for each sample of cells. Damage index thus ranged from 0 (completely undamaged: 100 cells  $\times$  0) to 400 (with maximum damage: 100 cells  $\times$  4) ([Collins, 2004](#)). The frequency of tailed cells, a DNA damage frequency indicator, was also calculated based on the number of cells with or without tails.

### 2.4. Statistical analysis

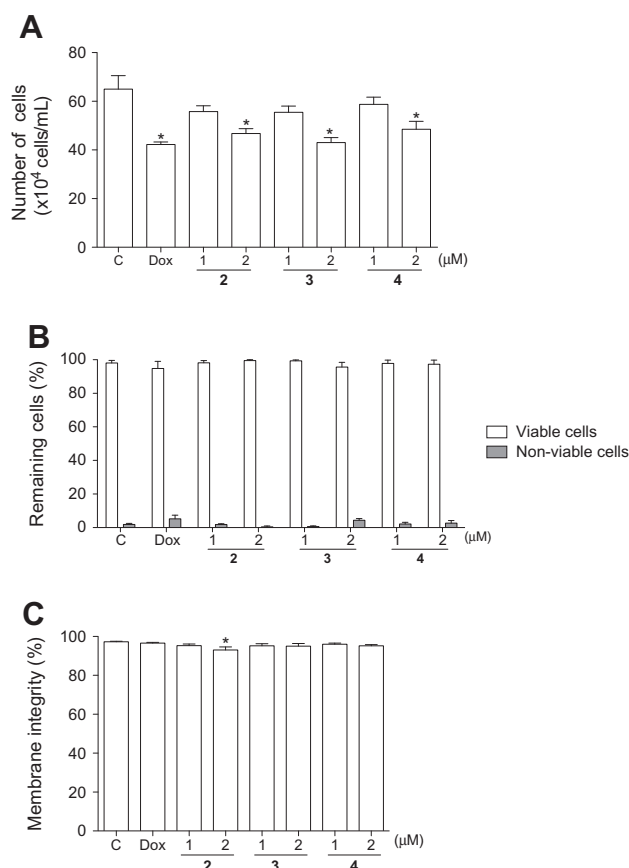
In order to determine differences among treatments, data were compared by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test ( $p < 0.05$ ) using the Graphpad program (Intuitive Software for Science, San Diego, CA). All studies were carried out in triplicate represented by independent biological evaluations.

## 3. Results

### 3.1. Antiproliferative effects of santonin derivatives

The indirect inhibitory growth effects of  $\alpha$ -santonin derivatives (2–4) on HL-60 cells were determined by MTT assay in a previous study ([Arantes et al., 2010, 2009](#)). The derivatives showed high activity, possessing IC<sub>50</sub> values in the range of 1.1–2.3  $\mu$ M on HL-60 cells. Regarding to normal cells (PBMC), IC<sub>50</sub> values ranged from 3.2 to 13.4  $\mu$ M and were less pronounced than those found in cancer cells.

As shown in [Fig. 2A](#), compounds 2, 3 and 4 caused reduction in HL-60 cell number in the concentration of 2  $\mu$ M after 24 h treatment and evaluation by trypan blue exclusion test ( $46.7 \pm 2.0$ ,  $43.0 \pm 2.1$  and  $48.5 \pm 3.3 \times 10^4$  cells/mL, respectively) when compared to control cells ( $65 \pm 5.5 \times 10^4$  cells/mL) ( $p < 0.05$ ), while no differences between the compounds were noticed ( $p > 0.05$ ). The positive control Dox also caused a significant reduction on viable cell population ( $42.2 \pm 1.0 \times 10^4$  cells/mL,  $p < 0.05$ ). Interestingly, though all compounds has decreased cell number after 24 h exposure, none of them altered viability of the remaining cells, since it was not noticed statistically significant differences in viable and non-viable cells in comparison to control ([Fig. 2B](#)). The cytotoxicity is not related to the membrane lysis of leukemia cells, since compounds 3 and 4 did not led to membrane disruption or increased fluorescence after ethidium bromide incorporation. The exception was the compound 2 (2  $\mu$ M), which induced a slight but significant decreasing in cells with intact membranes ( $93.0 \pm 1.6\%$ ,  $p < 0.05$ ) ([Fig. 2C](#)). Since sesquiterpene lactones are known inhibitors of enzymes and cellular processes, we investigated whether the inhibition of cell proliferation is related to DNA synthesis inhibition using the BrdU assay. This method



**Fig. 2.** Effects of the  $\alpha$ -Santonin derivatives 2, 3 and 4 (1 and 2  $\mu$ M) on HL-60 leukemia cell number (A), remaining cell viability (B) and determined by trypan blue exclusion test and membrane integrity analyzed using flow cytometry after 24 h exposure (C). Negative control was incubated with the vehicle used to dilute the molecules (DMSO 0.1%). Doxorubicin (0.6  $\mu$ M) was used as positive control (Dox). Results are expressed as mean  $\pm$  standard error of measurement (SEM) from three independent experiments. \* $p < 0.05$  compared by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test.

revealed that all compounds were able to reduce the BrdU incorporation, presenting the compound 4 the highest potential to diminish BrdU-positive cells in both dose tested (1  $\mu$ M and 2  $\mu$ M,  $28.5 \pm 2.2\%$  and  $28 \pm 1.9\%$ , respectively) in comparison to negative control ( $51.4 \pm 3.15\%$ ).

### 3.2. Santonin derivatives induced cell cycle arrest and DNA fragmentation

To define the mechanism responsible for the action of santonin derivatives involved on HL-60 cell death, cell-cycle distribution was assessed after 24 h and 48 h of treatment (Fig. 3A and B). A significant inhibition on HL-60 cell-cycle progression was observed within 24 h, where Dox ( $37 \pm 3.4\%$ ), compound 3 ( $7.6 \pm 0.5\%$  and  $9.0 \pm 0.9\%$ ) and 4 ( $9.0 \pm 0.9\%$  and  $8.6 \pm 9.6\%$ ) (1 and 2  $\mu$ M, respectively) caused an increasing of cells in G<sub>2</sub>/M phase when compared to untreated cells ( $3.4 \pm 0.5\%$ ). On the other hand, 48 h exposure provoked G<sub>2</sub>/M reduction [( $2.6 \pm 0.7\%$  and  $1.5 \pm 1.0\%$ ), ( $1.7 \pm 0.3\%$  and  $1.5 \pm 0.5\%$ ) and ( $0.6 \pm 0.2\%$  and  $1.0 \pm 0.8\%$ ), for compounds 2, 3 and 4, respectively] when compared to negative control ( $5 \pm 0.8\%$ ) (Fig. 3C,  $p < 0.05$ ), findings indicating time and concentration dependent activity of the molecules. Interestingly, only compound 2 at highest concentration was able to increase sub-G<sub>0</sub>/G<sub>1</sub> DNA content after 24 h ( $34 \pm 4.8\%$ , indicated in pink part) in comparison with control ( $13 \pm 1.3\%$ ) (Fig. 3D). However, after

48 h exposure,  $\alpha$ -santonin derivatives 3 and 4 also caused increasing on DNA fragmentation [( $45.3 \pm 1.2\%$  and  $91.0 \pm 2.0\%$ ) and ( $64.4 \pm 1.8\%$  and  $83.8 \pm 4.8\%$ ), respectively] in both concentrations tested (1  $\mu$ M and 2  $\mu$ M,  $p < 0.05$ ), though the compound 2 has increased DNA fragmentation only at 2  $\mu$ M ( $75 \pm 5.6\%$ ,  $p < 0.05$ , Fig. 3D).

### 3.3. Santonin derivatives induces cell death by apoptosis

To corroborate the suggestion the mechanism of action, we explored some hallmarks of apoptosis during a 24 h HL-60 cell exposure to the  $\alpha$ -santonin derivatives (2, 3 and 4).

#### 3.3.1. Acridine orange/ethidium bromide staining

For this purpose, HL-60 cells treated with the lactones 2, 3, and 4 were stained with AO/EB in order to discriminate cells undergoing necrosis or apoptosis. The compounds 2, 3 and 4 were able to reduce the number of viable cells at higher concentrations [2  $\mu$ M ( $77.3 \pm 1.5\%$ ,  $70.7 \pm 0.1\%$  and  $70.1 \pm 2.1\%$ )] and to expand the apoptosis level ( $20.5 \pm 1.6\%$ ,  $26.6 \pm 0.4\%$  and  $26.4 \pm 1.5\%$ ), respectively ( $p < 0.05$ ). On the other hand, compound 4 was the single concentration capable to decrease the number of viable cells at 1  $\mu$ M ( $84.1 \pm 1.5\%$ ) when compared to negative control ( $92.5 \pm 0.5\%$ ) ( $p < 0.05$ , respectively). At lowest concentrations, compounds 2, 3 and 4 also induced apoptosis ( $14.0 \pm 1.1\%$  and  $11.8 \pm 0.6\%$  and  $13.6 \pm 1.6\%$ , respectively) (Fig. 4,  $p < 0.05$ ), though in lower levels. The positive control (Dox, 0.6  $\mu$ M) reduced viable cells ( $60.0 \pm 7.3\%$ ) and increased apoptosis ( $36.2 \pm 4.8\%$ ).

When examined under light microscopy, control cells exhibited a typical non-adherent and round morphology, while derivatives-treated cells displayed chromatin condensation, nuclear fragmentation and shrinking in all concentrations tested (Fig. 4). Dox also induced cell reduction and nuclear disintegration.

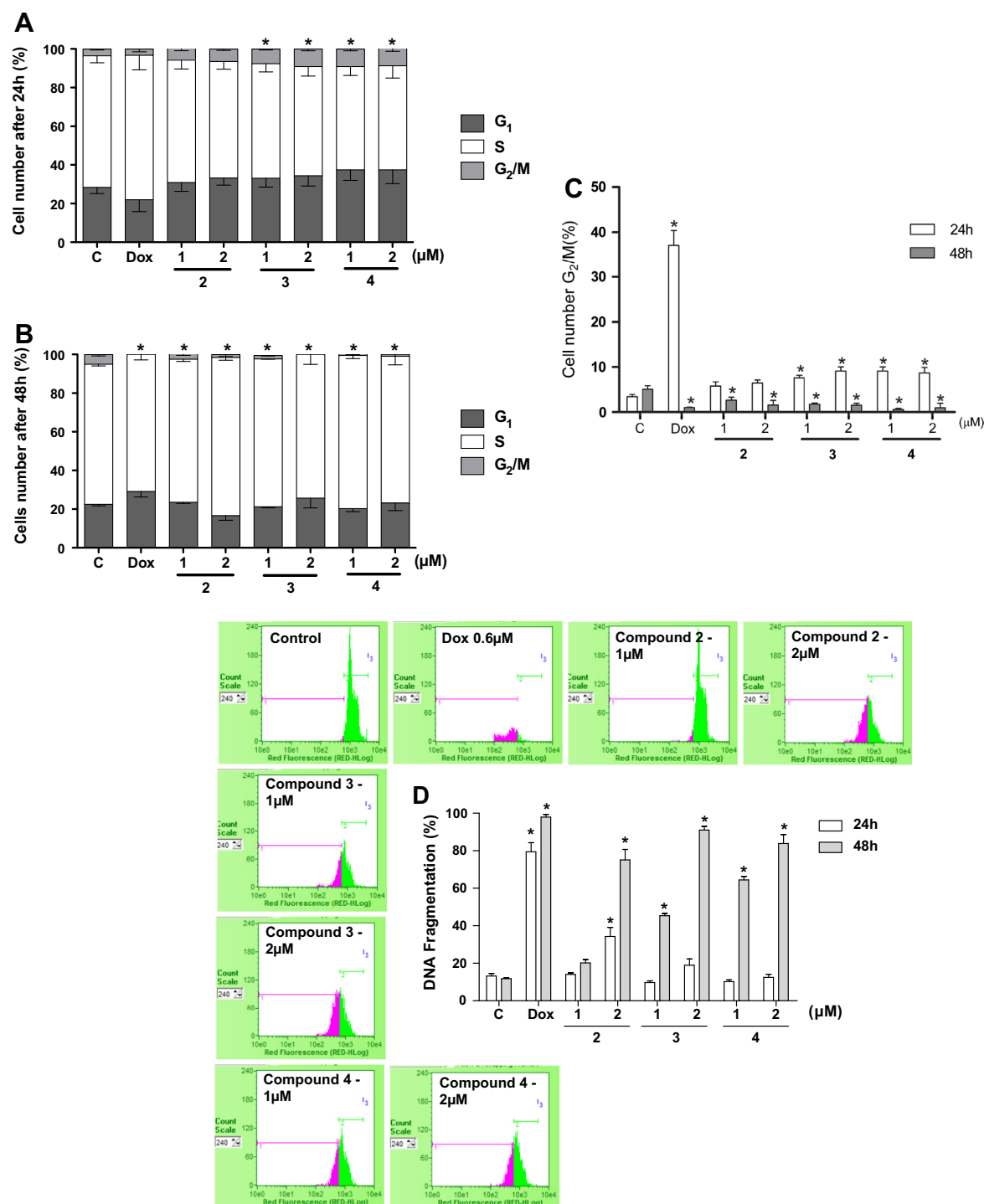
#### 3.3.2. Santonin derivatives induces phosphatidylserine externalization

Phosphatidylserine externalization was determined using Annexin V test as a marker of apoptosis. Annexin V, a 35 kDa Ca<sup>2+</sup> phospholipid-binding protein, binds to the phosphatidylserine on the outer layer of the plasma membrane with a high affinity due to loss of polarity whereas propidium iodide (PI) bind to cells that lost membrane integrity (Krysko et al., 2008). After 24 h exposure, compounds 2, 3 and 4 at 2  $\mu$ M were able to reduce cell viability ( $90.2 \pm 1.5\%$ ,  $89.5 \pm 1.6\%$  and  $86.7 \pm 2.7\%$ ), to induce early ( $7.5 \pm 0.8\%$ ,  $7.6 \pm 1.0\%$  and  $8.7 \pm 0.7\%$ ) and late apoptosis ( $0.8 \pm 0.1\%$ ,  $0.6 \pm 0.1\%$  and  $0.7 \pm 0.2\%$ ) and necrosis ( $1.6 \pm 0.3\%$ ,  $1.4 \pm 0.1\%$  and  $1.6 \pm 0.4\%$ ) on leukemia cells in comparison with control ( $92.5 \pm 0.6\%$ ,  $5.9 \pm 1.0\%$ ,  $0.2 \pm 0.1\%$  and  $0.4 \pm 0.1\%$ , respectively) (Fig. 5A,  $p < 0.05$ ). Meanwhile, Dox-treated tumor cells also revealed cell viability decreasing ( $50.5 \pm 0.2\%$ ), high levels of early apoptosis ( $47.5 \pm 0.3\%$ ) and necrosis ( $1.6 \pm 0.1\%$ ) following 24 h of treatment ( $p < 0.05$ ).

#### 3.3.3. Caspase cell activation

The main characteristic of cell undergoing apoptosis is the activation of caspases. The caspases can be categorized into initiator (8, 9 and 10) and executing caspases (3, 6 and 7) (Hanahan and Weinberg, 2011). At highest concentration, the compounds 2, 3 and 4 reduced cell viability ( $83.2 \pm 5.2\%$ ,  $83.4 \pm 6.6\%$  and  $76.3 \pm 8.5\%$ ) and increased the number of early ( $7.3 \pm 2\%$ ,  $5.8 \pm 2.5\%$  and  $9.1 \pm 4.1\%$ ) and late apoptosis cells ( $4.5 \pm 0.8\%$ ,  $5.0 \pm 0.7\%$  and  $4.8 \pm 0.5\%$ , respectively) in comparison with negative control ( $94.3 \pm 1.5\%$ , viable cells;  $1.7 \pm 0.9\%$ , early apoptosis and  $1.5 \pm 0.2\%$ , late apoptosis) ( $p < 0.05$ ) (Fig. 5B). Similarly, Dox also caused a significant cell viability decreasing ( $16.1 \pm 0.1\%$ ) and early apoptosis rising ( $83.2 \pm 0.1\%$ ).





**Fig. 3.** Effects of the  $\alpha$ -santonin derivatives 2, 3 and 4 (1 and 2  $\mu$ M) on HL-60 cell cycle evaluated by flow cytometry using propidium iodide, Triton X-100 and citrate. General view of the cell cycle (A and B), percentage of cell number in G<sub>2</sub>/M phase (C) and DNA fragmentation (D) after 24 h and 48 h of treatment. Negative control (C) was treated with the vehicle used for diluting the tested substance. Dox (0.6  $\mu$ M) was used as positive control (Dox). Results are expressed as mean  $\pm$  standard error of measurement (SEM) from three independent experiments. \* $p$  < 0.05 compared by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test.

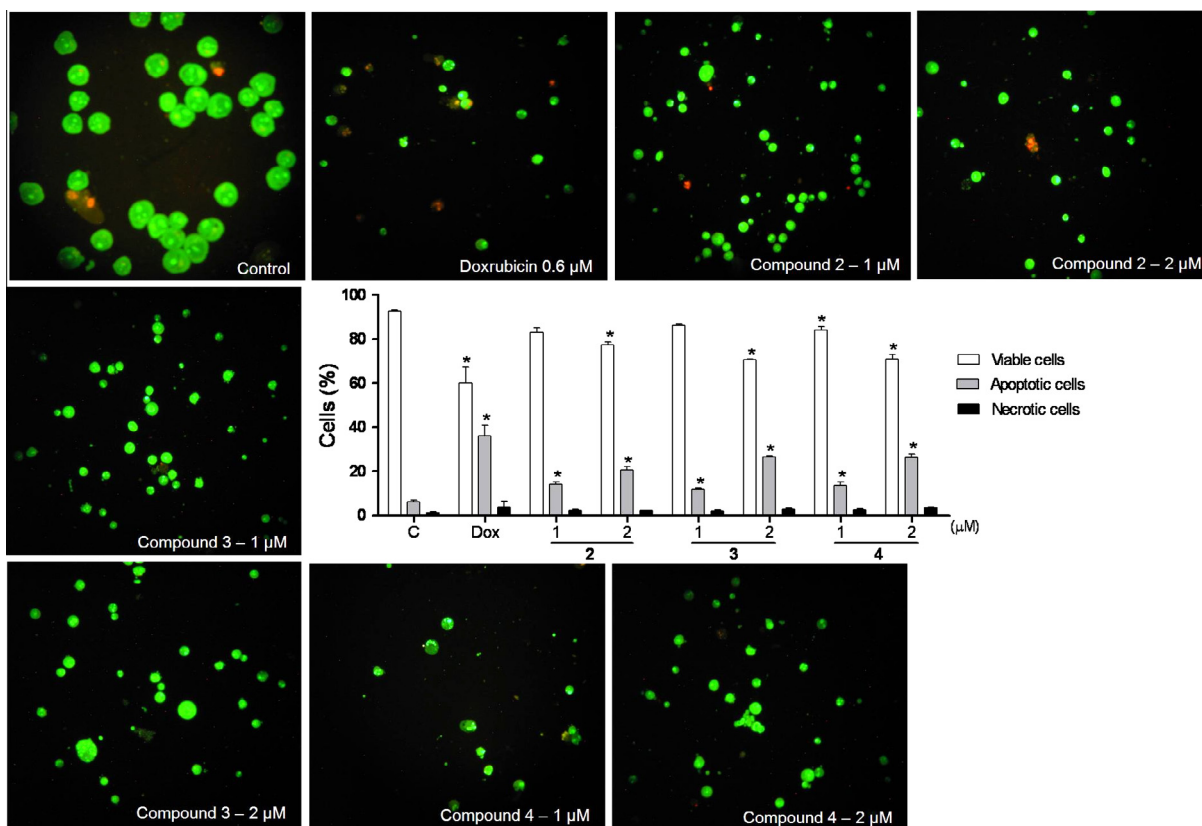
### 3.3.4. Mitochondrial membrane potential ( $\Delta\psi$ m)

Another early marker of the apoptotic process is the depletion of mitochondria membrane potential. In this work, none of the compounds evaluated in 24 h of treatment significantly alter the mitochondrial membrane potential ( $p$  > 0.05), suggesting that only the extrinsic pathway was activated within 24 h. However, in 48 h exposure, compound 4 (2  $\mu$ M) caused depolarization of mitochondrial membrane potential ( $37.3 \pm 4.6\%$ , Fig. 5C) when compared to negative control ( $4.7 \pm 0.6\%$ ,  $p$  < 0.05). Dox, positive control, cause

intense membrane depolarization after 24 h ( $44.0 \pm 2.3\%$ ) and 48 h ( $46.9 \pm 5.4\%$ ) of incubation.

### 3.4. Comet assay

The DNA damage induced by the  $\alpha$ -santonin derivatives was evaluated in human mononuclear cells. DNA damages were not detected with the concentrations tested ( $p$  > 0.05, data not shown).



**Fig. 4.** Cell death pattern determined by acridine orange/ethidium bromide (AO/EB) after 24 h exposure. Control (C) was treated with the vehicle used for diluting the tested substance. Doxorubicin (0.6  $\mu$ M) was used as positive control (Dox). Results are expressed as mean  $\pm$  standard error of measurement (SEM) from three independent experiments. \* $p < 0.05$  compared by one-way analysis of variance (ANOVA) followed by the Newman–Keuls test.

#### 4. Discussion

Sesquiterpene lactones (SLs) are plant-derived compounds often used in traditional medicine against several human diseases such as inflammation and cancer (Ghantous et al., 2010). Previous researches showed no cytotoxic activity of the  $\alpha$ -santonin molecule, even at high concentrations (100  $\mu$ M) (Kim et al., 2002; Konaklieva and Plotkin, 2005). Then, we designed three cytotoxic sesquiterpene lactones based on  $\alpha$ -santonin (Arantes et al., 2009; 2010) with activity on different cancer cell lines and low toxicity on PBMC. In this work, we propose the mechanism responsible for this cytotoxicity using the HL-60 cell line as experimental model and the compounds tested (1 and 2  $\mu$ M) after 24 h of treatment.

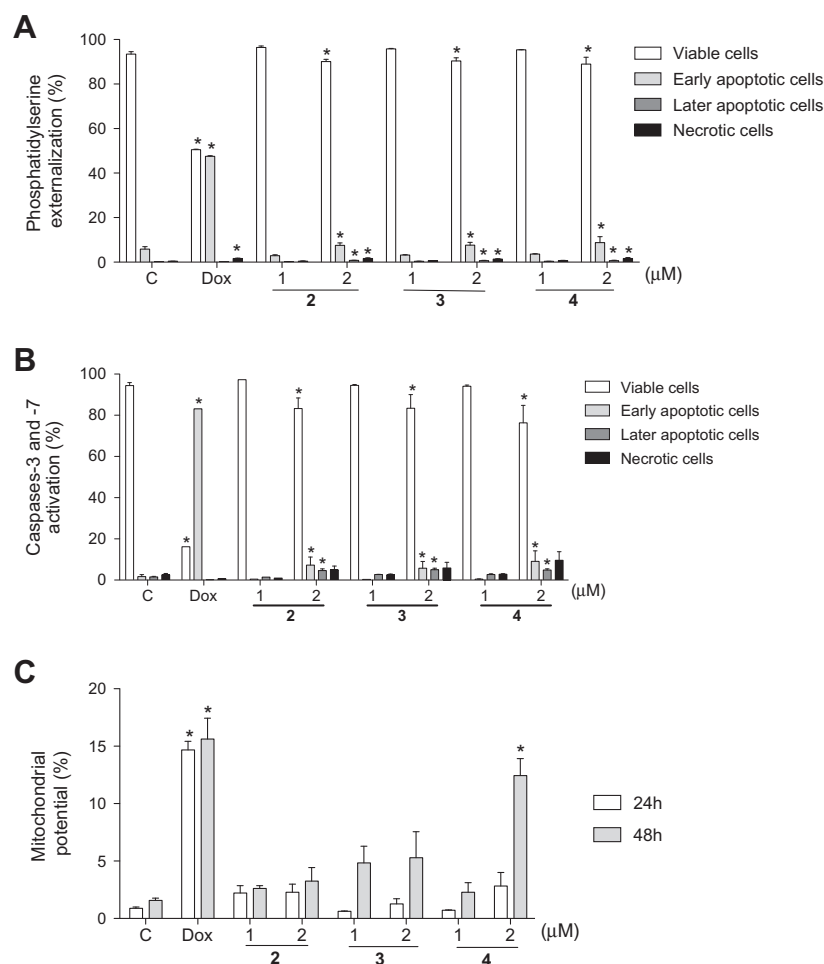
Initially, we showed that the antiproliferative potential of the  $\alpha$ -santonin derivatives is not related to direct membrane damages, since the trypan and propidium iodide exclusion techniques did reveal membrane permeability of remaining cells. In fact, it is possible that apoptosis or other process might have already compromised cell proliferation, but membrane integrity is still maintained (Kepp et al., 2011). We previously reported that these derivatives did not produce cell membrane disruption of mouse erythrocytes (Arantes et al., 2010).

Some studies have been pointed that SLs inhibit tumor growth by selective alkylation of growth-regulatory biological macromolecules, such as DNA and key enzymes, which control cell division, thereby inhibiting a variety of cellular functions, which leads cells into apoptotic death (Fernandes et al., 2008; Rozenblat et al., 2008). Herein, all molecules reduced BrdU incorporation by HL-60 treated cells, suggesting inhibition of DNA synthesis. Other SLs caused inhibition of DNA synthesis by BrdU test such as

enhydin, uvedalin and sonchifolin (Siriwan et al., 2011). The apoptotic effect of these SLs is associated with caspase 3/7 activation.

All compounds (2, 3 and 4) increased cell death with morphological characteristics of apoptosis and reduced the number viable cells at 2  $\mu$ M, whose concentration decreased plasma membrane integrity as seen by trypan blue test. Furthermore, AO/BE staining analysis after 24 h of incubation revealed treated cells displaying typical apoptotic and necrotic features, including reduction in cell volume, intense karyorrhexis, pyknotic nuclei typical of necrotic processes and signs of plasma membrane destabilization, which indicates quick activation of apoptosis pathways that culminate in secondary necrosis activation (de Bruin and Medema, 2008). Dose-dependent regulation of cellular processes is one of the most important characteristics of signaling molecules naturally occurring in cells. Therefore, depending on the concentration used, many different processes may be influenced and/or altered. Indeed, treated cells displayed apoptotic features at concentrations as low as 1  $\mu$ M with an increase of necrotic cells at 2  $\mu$ M, probably as a result of a later apoptosis stage.

To elucidate the probable mechanism by the antiproliferative effects of  $\alpha$ -santonin derivatives (B–D), we first examined whether inhibition of cell viability by the SLs was associated with changes in cell cycle progression. Compounds 3 and 4 produced cell cycle arrest at G<sub>2</sub>/M transition. The cell cycle arrest reflects a requirement to repair cell damages; if not repaired, apoptotic mechanisms are often activated (Rozenblat et al., 2008). Other SLs are known to arrest cell cycle. Thus, the molecules 6-O-angeloylenolin and dehydrocostuslactone induced cell-cycle arrest and apoptosis in human nasopharyngeal and ovarian cancer cells, respectively (Su et al., 2011). Tomentosin (36 and 54  $\mu$ M) and Inuviscolide (36 and 72  $\mu$ M) caused cell cycle arrest at G<sub>2</sub>/M, phosphatidylserine



**Fig. 5.** Flow cytometry analysis of the death pattern in HL-60 cells treated with  $\alpha$ -santonin derivatives 2, 3 and 4 (1 and 2  $\mu$ M) performed with Annexin V-PE and 7-amino-actinomycin-D (phosphatidylserine externalization) (A) and FLICA™ solution (activation of caspases-3 and -7) after 24 h (B) and rhodamine 123 (mitochondrial transmembrane potential) at 24 h and 48 h exposure (C). Negative control (C) was treated with the vehicle used for diluting the tested substance. Doxorubicin (0.6  $\mu$ M) was used as positive control (Dox). Results are expressed as mean  $\pm$  standard error of measurement (SEM) from three independent experiments. \* $p$  < 0.05 compared by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test.

exposition and caspase-3 activation in SK-28 cells (human melanoma).

$G_0/G_1$  subpopulation represented DNA fragmentation on flow cytometry cell cycle assay (Krysko et al., 2008). In this event, only the compound 2 at highest concentration was able to cause DNA fragmentation following 24 h exposure. On the other hand, after 48 h all compounds induced DNA fragmentation. Internucleosomal DNA fragmentation is a nuclear feature of apoptosis and double-stranded DNA disintegration is attributed to caspases (Huerta et al., 2007), cysteine aspartate-specific proteases synthesized as zymogens that cleave different proteins (Krysko et al., 2008). These enzymes are involved in two different apoptotic pathways: the intrinsic and extrinsic pathways, each possessing your specific initiator enzymes (caspase-9 and -8, respectively). Both pathways can activate executor caspases (caspase-3, -6 and -7), being caspase-3 the major effector caspase that predominantly triggers laminin and nuclear mitotic apparatus collapse (Hanahan and Weinberg, 2000, 2011; Widlak and Garrard, 2009). While necrotic cells present random DNA fragmentation, the caspases produce a pattern damaged DNA with intervals of 180–200 base pairs (Krysko et al., 2008). Herein, all molecules assessed induced caspases activation, with intensification in early and late apoptotic processes.

The phosphatidylserine externalization induced at 2  $\mu$ M is an additional finding that corroborates activation of pathways

suggestive of apoptosis. Loss of membrane polarity, caused by translocation of phosphatidylserine from the inner to outer plasma membrane, thereby exposing phosphatidylserine for external environment, stimulates recognition and engulfment of dying cells by macrophages and other antigen presenting cells (Kroemer et al., 1997).

In order to improve comprehension about cell signaling, it was performed a mitochondrial depolarization analyses. Apoptosis stimulation by the mitochondrial via starts with a pore formation in the external membrane mitochondrial and release of cytochrome c, with permeability changes and mitochondrial membrane potential collapse. This failure can be measured by cationic lipophilic fluorochromes, such as rhodamine 123 (de Thonel and Eriksson, 2005). Within 24 h of treatment,  $\alpha$ -santonin derivatives did not induce modifications in mitochondrial potential. However, following 48 h, the compound 4 induced mitochondrial depolarization, an indicative of apoptosis intrinsic pathway activation commonly caused in reaction to DNA damage, absence of survivor factors and several types of cellular stress. Whereas caspase-8 was activated, it is likely that it had been occurred convergence into apoptosis intrinsic pathway downstream from the extrinsic route. Costunolide, a sesquiterpene lactone structure close to  $\alpha$ -santonin, induces  $G_2/M$  cell cycle arrest and cause apoptosis on several cell tumor lines through extrinsic pathway before intrinsic

activation, leading to the caspase-8 Bid stimulation, a pro-apoptotic protein belonging to BCL-2 family that also activates mitochondrial pathways (Liu et al., 2011). This indirectly mitochondrial involvement could explain a longer extensive interval required (48 h) by  $\alpha$ -santonin derivatives 2 and 4 to instigate cell damage, which culminates with mitochondrial depolarization and DNA destabilization (de Thonel and Eriksson, 2005; Liu et al., 2011; Choi et al., 2012; Guzman and Jordan, 2005).

Sesquiterpene lactones have a privileged selectivity for tumor cells (Ghantous et al., 2010). Previously, compounds 2, 3 and 4 were tested against normal cells (PBMC) and showed low toxicity (Arantes et al., 2010). Here, the same ones were tested in alkaline comet assay to analyze their ability to cause DNA alkylation on PBMC. None of compounds were able to DNA disruptions in both concentrations tested (data not shown). Similarly, it was displayed that Parthenolide, another sesquiterpene lactone, selectively kill primitive leukemia cells without affecting normal stem and progenitor hematopoietic cells (Guzman and Jordan, 2005).

## 5. Conclusions

We propose the extrinsic pathway of apoptosis as the central via involved in cell death induced by these  $\alpha$ -santonin derivatives. The compounds showed low toxicity effects on normal and higher cytotoxic on tumor cells, a very desired advantage in new lead anticancer chemicals to overwhelmed adverse effects due to therapeutic narrow window, pharmacological multiple resistance and morphological and physiological similarities between transformed and normal cells.

## Conflict of interest statement

The authors have declared that there is no conflict of interest.

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